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Article

Leishmania Evades Host Immunity by Inhibiting Antigen Cross-Presentation through Direct Cleavage of the SNARE VAMP8

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SUMMARY

During phagocytosis, microorganisms are taken up by immune cells into phagosomes. Through membrane-trafficking events mediated by SNARE proteins, phagosomes fuse with lysosomes, generating degradative phagolysosomes. Phagolysosomes contribute to host immunity by linking microbial killing within these organelles with antigen processing for presentation on MHC class I or II molecules to T cells. We show that the intracellular parasite *Leishmania* evades immune recognition by inhibiting phagolysosome biogenesis. The *Leishmania* cell surface metalloprotease GP63 cleaves a subset of SNAREs, including VAMP8. GP63-mediated VAMP8 inactivation or *Vamp8* disruption prevents the NADPH oxidase complex from assembling on phagosomes, thus altering their pH and degradative properties. Consequently, the presentation of exogenous *Leishmania* antigens on MHC class I molecules, also known as cross-presentation, is inhibited, resulting in reduced T cell activation. These findings indicate that *Leishmania* subverts immune recognition by altering phagosome function and highlight the importance of VAMP8 in phagosome biogenesis and antigen cross-presentation.

INTRODUCTION

Phagocytosis, the process by which cells of the immune system such as macrophages and dendritic cells engulf microorganisms at sites of infection, leads to the formation of phagosomes, where microbes are killed and processed for antigen presentation (Botelho and Grinstein, 2011; Boulais et al., 2010; Joffre et al., 2012). Because phagosomes play a key role in both innate and adaptive immunity, the functional properties of these organelles and the molecular mechanisms regulating their interactions with pathogens have been the attention of a large number of studies in the last 25 years. These studies have highlighted the diversity of molecular strategies used by a variety of microorganisms to interfere with the maturation of phagosomes into phagolysosomes, a process referred to as phagolysosome biogenesis (Alix et al., 2011; Flannagan et al., 2009). Phagolysosome biogenesis is essential for the acquisition of the microbicidal properties required for the killing of microbes (Desjardins et al., 2005; Jutras and Desjardins, 2005). This process is driven by complex sets of membrane-trafficking events involving soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein-regulated sequential fusion events between phagosomes and intracellular organelles, including early endosomes, late endosomes, and lysosomes (Stow et al., 2006). These interactions allow the coordinated transfer of sets of hydrolases, as well as the acquisition of the oxidative machinery, involved in the killing of microorganisms and the processing of some of their proteins for antigen presentation on MHC class II molecules. Furthermore, the interaction between phagosomes and subregions of the endoplasmic reticulum plays a key role in the contribution of this organelle to cross-presentation, the process by which exogenous microbial antigens are presented on MHC class I molecules (Campbell-Valois et al., 2012; Jutras and Desjardins, 2005).

The protozoan *Leishmania* parasitizes phagocytic cells and causes a spectrum of human diseases known as leishmaniases. Once inoculated by sand fly vectors into mammals, promastigotes are taken up by macrophages. We previously reported that one mechanism used by promastigotes to evade the microbicidal consequences of phagocytosis is the inhibition of phagolysosome biogenesis (Desjardins and Descoteaux, 1997). Hence, promastigotes are internalized in phagosomes that poorly interact with late endosomes and lysosomes and display a delayed recruitment of LAMP-1 (Dermine et al., 2000; Scianimanico et al., 1999; Späth et al., 2003). Lipophosphoglycan (LPG), the major promastigote surface glycolipid, is responsible

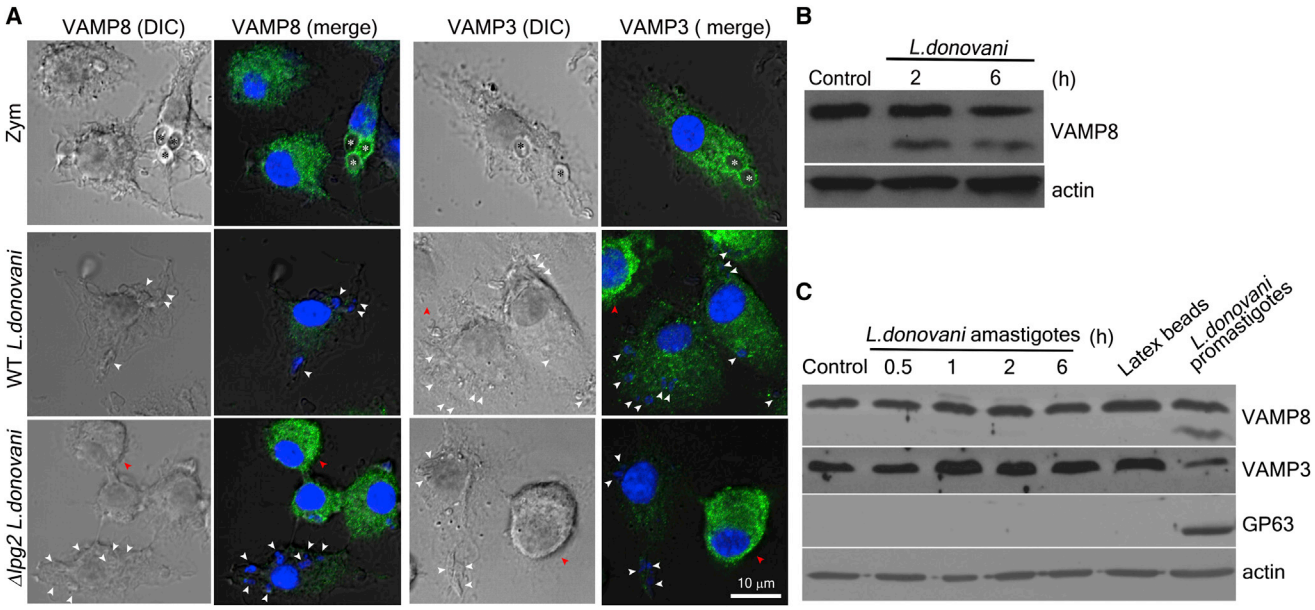

Figure 1. *Leishmania* Promastigotes Target Host Cell SNAREs

(A) Confocal microscopy images of BMMs 2 hr after internalization of Zym, WT, or Δ*lpg2 L. donovani* promastigotes. VAMP3 and VAMP8 are in green; nuclei are in blue. Asterisks show phagosomes containing Zym, and white arrowheads show phagosomes containing *L. donovani*. Red arrowheads show uninfected cells. DIC, differential interference contrast.

(B) VAMP8 and actin in lysates of BMMs infected with *L. donovani* promastigotes for the indicated time points.

(C) VAMP8, VAMP3, GP63, and actin in lysates of BMMs infected with *L. donovani* amastigotes for the indicated time points. Controls consisted of BMMs infected for 2 hr with *L. donovani* promastigotes or with latex beads. Similar results were obtained in three independent experiments. See also Figure S1.

for this inhibition (Desjardins and Descoteaux, 1997; Lodge and Descoteaux, 2008). LPG inserts itself in the phagosome membrane where it destabilizes lipid microdomains (Dermine et al., 2005), thereby impairing processes required for the generation of a microbicidal compartment within macrophages. One consequence of LPG-mediated microdomain disorganization is the exclusion of the membrane fusion regulator synaptotagmin V (Syt V) from the phagosome early after infection. This in turn abrogates recruitment of the V-ATPase and impedes phagosome acidification (Vinet et al., 2009). Targeting of the phagosome fusion machinery thus represents an efficient way for *Leishmania* promastigotes to create an intracellular niche favorable to the establishment of infection.

These findings led us to seek further insight into the impact of *Leishmania* promastigotes on the host cell fusion machinery that controls phagosome maturation and function. Here, we provide evidence that *Leishmania* promastigotes impair phagosome maturation and antigen cross-presentation through the proteolytic cleavage of SNAREs.

RESULTS

Leishmania Promastigotes Exclude SNAREs from Phagosomes

Infection with the intracellular pathogen *Leishmania* is initiated when promastigotes are engulfed by phagocytic cells at the site of inoculation. The main survival strategy of this parasite is then to inhibit phagolysosome biogenesis. An important aspect of the molecular mechanisms enabling this inhibition relies on the exclusion of Syt V, a key regulator of membrane fusion, from the phagosomal membrane (Vinet et al., 2009). In addition to Syt V, the phagosome proteome contains several soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Boulais et al., 2010). Hence, it is conceivable that multiple aspects of phagolysosome biogenesis, crucial for the acquisition of the microbicidal properties of this organelle and its ability to properly process proteins for antigen presentation, are regulated by a series of membrane fusion regulators such as SNAREs. To determine whether *Leishmania* impairs the recruitment of other SNAREs, we infected bone marrow-derived macrophages (BMMs) with *L. donovani* promastigotes and assessed the intracellular localization of VAMP3 and VAMP8, two SNAREs known to be present on phagosomes (Furuta et al., 2010; Kay et al., 2006; Murray et al., 2005). Immunofluorescence analyses at the confocal microscope show that both VAMP3 and VAMP8 were present on zymosan (Zym)-containing phagosomes at 2 and 6 hr after the initiation of phagocytosis (Figure 1A). In contrast, these two SNAREs were absent from the majority of phagosomes containing wild-type (WT) *L. donovani* promastigotes (Figure 1A). Because the virulence glycolipid LPG is the only known *Leishmania* factor responsible for phagosome remodeling (Dermine et al., 2001; Desjardins and Descoteaux, 1997; Lodge et al., 2006; Scianimanico et al., 1999; Vinet et al., 2009), we tested whether this molecule was involved in the exclusion of VAMP3 and VAMP8 from *Leishmania*-containing phagosomes. Our results show that VAMP3 and VAMP8 are still excluded from phagosomes containing the LPG-defective Δ*lpg2 L. donovani* mutant, indicating

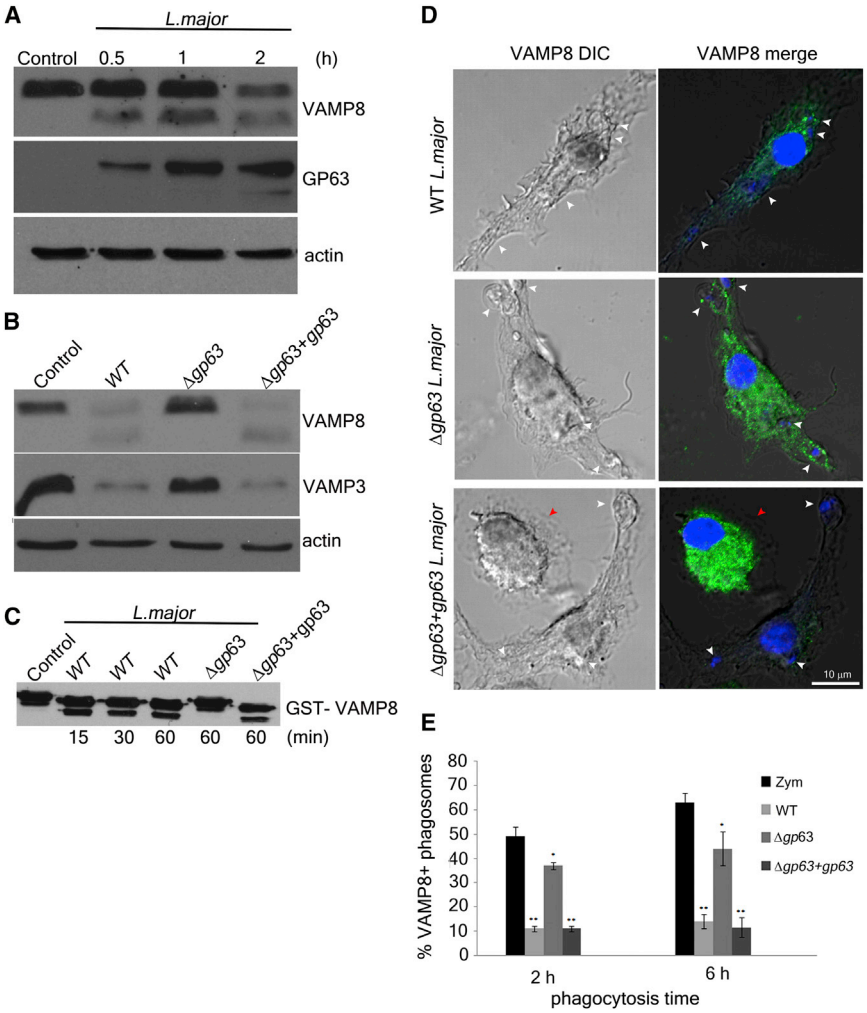

Figure 2. GP63 Mediates SNARE Cleavage in Infected BMMs

(A) Kinetics of VAMP8 cleavage by *L. major* and GP63 accumulation in lysates of BMMs was assessed by immunoblot analysis.

(B) VAMP8 and VAMP3 in lysates from BMMs infected for 2 hr with WT, Δ*gp63*, or Δ*gp63+gp63 L. major* promastigotes.

(C) GST-VAMP8 was incubated with *L. major* promastigotes, and cleavage was assessed by immunoblot analysis.

(D) Confocal microscopy images of BMMs infected for 2 hr with WT, Δ*gp63*, or Δ*gp63+gp63 L. major* promastigotes. VAMP8 is in green; nuclei are in blue. White arrowheads show phagosomes containing *L. major*. Red arrowheads show uninfected cells.

(E) Quantification of VAMP8-positive phagosomes at 2 and 6 hr after infection. Data are presented as the mean ± SD of three independent experiments. *p ≤ 0.05; **p ≤ 0.005. See also Figure S2.

that LPG is not responsible for the phagosomal exclusion of both SNAREs (Figure 1A). This finding pointed toward the existence of an unsuspected mechanism used by *Leishmania* promastigotes to remodel their phagosomes. Intriguingly, we observed that VAMP3 and VAMP8 were not only excluded from phagosomes but that the intensity of the labeling for these proteins was also reduced throughout the cell in BMMs infected with *L. donovani* promastigotes compared to BMMs that had internalized Zym, or to uninfected BMMs (Figure 1A). Western blotting analyses confirmed that the amount of both proteins was much lower in infected cells. Furthermore, the appearance of a second band of lower molecular mass suggested that VAMP8 was actually cleaved in infected cells (Figure 1B). Thus, the cleavage of key proteins involved in membrane trafficking may be part of a strategy used by *Leishmania* promastigotes to remodel their intracellular niche.

GP63 Mediates the Cleavage of SNAREs

The cleavage of VAMP8 in *Leishmania*-infected BMMs is reminiscent of that of SNAREs catalyzed by the zinc-dependent metalloproteases botulinum neurotoxins and antarease from scorpion venom (Fletcher et al., 2010; Montal, 2010). We therefore investigated whether a *Leishmania* protease is involved in VAMP8 cleavage. The *Leishmania* genome encodes several proteases (Ivens et al., 2005) including the surface GPI-anchored GP63, a zinc-dependent metalloprotease expressed at high levels in promastigotes (Etges et al., 1986). GP63 is a virulence factor that contributes to the pathogenesis of *Leishmania* by cleaving molecules controlling a variety of host defense mechanisms (Contreras et al., 2010; Corradin et al., 1999; Gomez et al., 2009; Hallé et al., 2009; Jaramillo et al., 2011; Joshi et al., 2002). During the differentiation of promastigotes into amastigotes, GP63 is downregulated (Figure S1 available online). Interestingly, we did not observe cleavage of either VAMP8 or VAMP3 in BMMs infected with *L. donovani* amastigotes, and this corresponds to the absence of GP63 (Figure 1C). These findings lent support to the possibility that GP63 was responsible for the cleavage of VAMP8 and VAMP3 in BMMs infected with promastigotes. The availability of a Δ*gp63* mutant in *L. major* allowed us to directly test this hypothesis (Joshi et al., 2002). We first showed that, similar to *L. donovani*, infection with *L. major* promastigotes resulted in a cleavage of VAMP8 that was detectable as early as 30 min postinfection (Figure 2A). Remarkably, VAMP8 remained intact in cells infected with the Δ*gp63* mutant (Figure 2B). The ability to cleave VAMP8 was regained when cells were infected with the add-back mutant (Δ*gp63+gp63 L. major*) promastigotes. VAMP3 was also degraded in a GP63-dependent manner (Figure 2B). By incubating purified glutathione S-transferase (GST)-tagged VAMP8 with *L. major* promastigotes, we showed that GP63 directly cleaves VAMP8 (Figure 2C). Collectively, these results identify GP63 as the parasite molecule responsible for the cleavage of host SNAREs during infection (Figures 2 and S2). Using confocal microscopy, we confirmed that phagosomal exclusion of VAMP8 (Figures 2D and 2E) and VAMP3 (data not shown) is

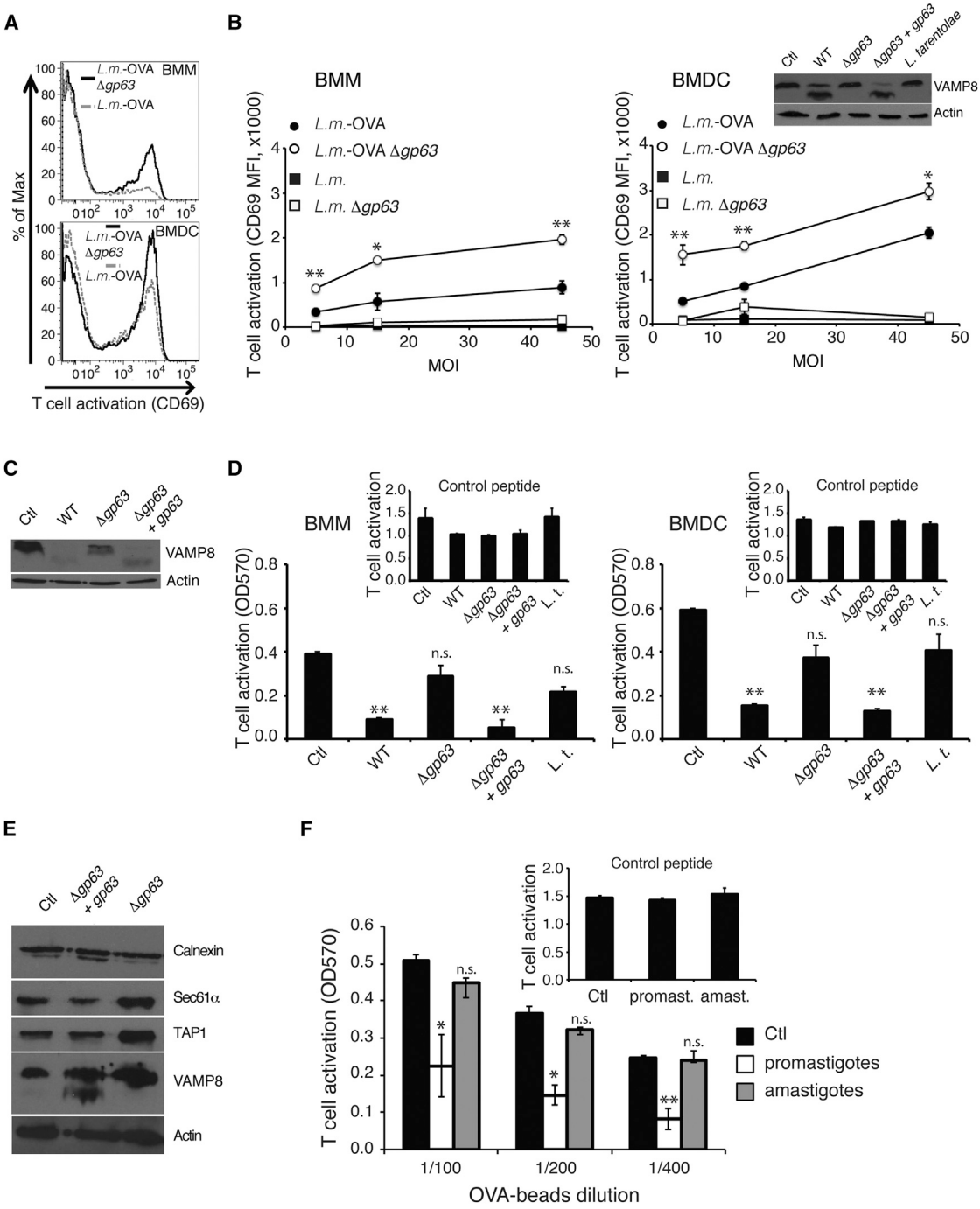

Figure 3. *Leishmania* Promastigotes Inhibit Cross-Presentation in a GP63-Dependent Manner

(A) Representative FACS histograms of CD8 OT-I T cell CD69 expression after culture with BMMs or BMDCs infected with WT or Δ*gp63 L. major*-OVA promastigotes. % of Max., percentage of maximum.

(B) Cells infected with WT or Δ*gp63 L. major*-OVA promastigotes or with nontransfected parasites were incubated with CD8 OT-I T cells, and cross-presentation was assessed by CD69 expression. Right upper-panel shows VAMP8 and actin in noninfected BMDCs or infected with WT, Δ*gp63*, Δ*gp63+gp63 L. major*, or *L. tarentolae* promastigotes. Ctl, control.

(C) VAMP8 in latex bead-containing phagosomes isolated from noninfected cells (Ctl) or infected with WT, Δ*gp63*, or Δ*gp63+gp63* promastigotes.

(D) Cells were infected with WT, Δ*gp63*, Δ*gp63+gp63 L. major*, or *L. tarentolae* promastigotes or left untreated (Ctl). OVA-coated beads or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) was added, and cross-presentation and MHC I expression were evaluated using the B3Z T cell hybridoma.

(E) Immunoblot analysis of molecules involved in the MHC class I machinery (calnexin, Sec61, TAP1) and VAMP8 in noninfected cells (Ctl) or cells infected with Δ*gp63* or Δ*gp63+gp63 L. major* promastigotes.

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GP63 dependent. Our results thus far highlight a pathogenesis mechanism used by *Leishmania* parasites to control biogenesis of the parasitophorous vacuole, whereby GP63 targets SNAREs that regulate vesicular trafficking to and from phagosomes.

Leishmania Impairs Antigen Cross-Presentation through the Action of GP63

In addition to the killing of the parasite, the processing of *Leishmania* proteins for antigen presentation on both MHC class II molecules and MHC class I molecules during cross-presentation is a highly regulated process that requires molecular properties acquired during phagolysosome biogenesis (Jutras and Desjardins, 2005). Although the precise molecular mechanisms involved in the processing of *Leishmania* proteins for cross-presentation are still poorly understood, it is assumed that parasite proteins are proteolytically cleaved in maturing phagosomes, generating peptides that can either be directly loaded on MHC class I molecules in phagosomes or translocated to the cytoplasm for further processing by the proteasome (Bertholet et al., 2005, 2006). Hence, trafficking events involved in phagolysosome biogenesis are likely to play key roles in cross-presentation during *Leishmania* infection. In that context, we elected to study whether the GP63-dependent cleavage of SNAREs observed during infection alters key immune processes such as cross-presentation in BMMs and BMDCs (bone marrow-derived dendritic cells).

To test this hypothesis in the context of *Leishmania* infection, we produced WT *L. major* and Δ*gp63 L. major* expressing ovalbumin (OVA) (*L. major*-OVA) to measure the cross-presentation of the OVA antigen on MHC class I molecules. BMMs and BMDCs were infected with promastigotes before the addition of OVA-specific $CD8^+$ OT-I T cells, and cross-presentation was assessed through the expression of the early T cell activation marker CD69. Negative controls consisted of *L. major* promastigotes not expressing OVA. Our results showed that cross-presentation was significantly higher in BMMs and BMDCs infected with Δ*gp63 L. major*-OVA promastigotes compared to cells infected with WT parasites (Figures 3A and 3B), demonstrating that GP63 endows these parasites with the ability to alter cross-presentation. A key question that arose at this point was whether the action of GP63 was restricted to phagosomes containing parasites or if the whole cellular machinery of cross-presentation was altered. We addressed this issue by showing that VAMP8 present on latex bead-containing phagosomes formed in cells infected with WT and Δ*gp63*+*gp63 L. major* promastigotes was also cleaved (Figure 3C). This result allowed us to test whether the cleavage of VAMP8 by GP63 is sufficient to alter cross-presentation by measuring the presentation of the SIINFEKL peptide following the internalization of OVA-coated latex beads in infected cells. Indeed, cross-presentation from OVA-coated beads was greatly diminished in BMMs and BMDCs infected with WT promastigotes compared to uninfected cells or cells infected with the Δ*gp63* mutant (Figure 3D). Infection with the GP63 add-back parasites (Δ*gp63* + *gp63*) restored the ability of the Δ*gp63* mutant to inhibit cross-presentation, highlighting the global effect of GP63 on this immune process. Interestingly, the nonpathogenic parasite *L. tarentolae*, in which GP63 is inactive (Campbell et al., 1992), was unable to cleave VAMP8 and to inhibit cross-presentation (Figures 3B and 3D). The inhibition of cross-presentation by *L. major* promastigotes was not caused by a decreased MHC class I expression, as shown by the SIINFEKL peptide-loading control experiment (Figure 3D), or by a defect in phagocytosis (Figure S3). Furthermore, infection with *Leishmania* promastigotes had no noticeable effect on the phagosomal recruitment of molecules involved in the antigenic peptide loading on MHC class I (Figure 3E). Importantly, amastigotes, in which GP63 is down modulated (Figure 1C), did not inhibit cross-presentation (Figure 3F). Collectively, these results show that a microbial molecule, GP63, directly impairs the ability of infected cells to cross-present exogenous peptides on MHC class I molecules. These results also support the hypothesis that *Leishmania* promastigotes transiently alter antigen cross-presentation by the degradation of a restricted set of molecules including SNAREs. Thus far, our study also indicates that the well-established latex bead system can be used to decipher the molecular mechanisms enabling GP63 to cleave VAMPs and the potential role played by these proteins in the regulation of cross-presentation.

VAMP8 Is Required for Antigen Cross-Presentation

Thus far, our results suggest that the cleavage of SNAREs by GP63 might be responsible for the alteration of cross-presentation in *Leishmania*-infected cells. Whereas VAMP3 regulates cytokine secretion at the phagocytic cup (Murray et al., 2005), little is known about the role of VAMP8 in phagosome function. However, VAMP8 is present on late endosomes where it controls homotypic fusion between these organelles (Pryor et al., 2004). Considering the importance of the regulation of fusion events during phagolysosome biogenesis, including phagosome-late endosome fusion (Desjardins et al., 1997), we investigated whether VAMP8 is required for cross-presentation. To this end, we used BMMs and BMDCs from VAMP8 null mice (Wang et al., 2004). Using our OVA-coated bead assay, we observed a decrease of around 50% in the level of cross-presentation in $Vamp8^{-/-}$ BMDCs and BMMs compared to WT cells (Figure 4A). This inhibition was not the consequence of a reduced MHC class I expression (Figure 4A, inset) or due to a defect in phagocytosis (Figure S4A) in $Vamp8^{-/-}$ cells. In addition, the absence of VAMP8 did not significantly affect the recruitment of the machinery involved in the antigenic peptide loading on MHC class I to phagosomes (Figure S4B). To determine whether the effect of GP63-mediated cleavage of VAMP8 on cross-presentation was specific, we assessed the impact of GP63 and VAMP8 on MHC class II-restricted antigen presentation in BMDCs. Interestingly, we found that this process was also inhibited by GP63 (Figure S4C). However, in contrast to cross-presentation, MHC class II-restricted antigen presentation was normal or even slightly superior in the absence of VAMP8 (Figure S4D). These

(F) BMDCs were infected with WT *L. donovani* promastigotes (promast.) or amastigotes (amast.), or left untreated (Ctl). OVA-coated beads or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) was then added. Cross-presentation and MHC I expression were evaluated using B3Z T cell hybridoma. Data are presented as the mean ± SEM of one experiment representative of at least two independent experiments. *$p \leq 0.05$; **$p \leq 0.01$. See also Figure S3.

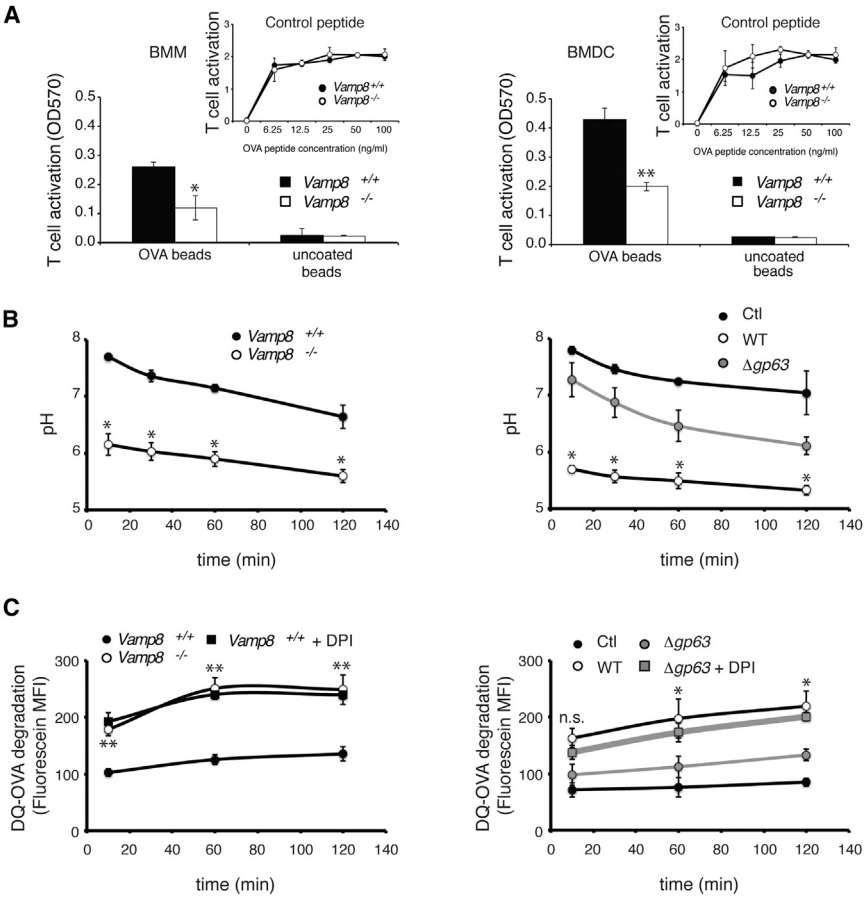

Figure 4. VAMP8 Regulates Antigen Cross-Presentation through the Control of Phagosome Acidification and Antigen Degradation

(A) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMMs or BMDCs were fed with OVA-coated beads or SIINFEKL peptide (upper graph). Cross-presentation and MHC I expression were evaluated using B3Z T cell hybridoma.

(B) *Vamp8*$^{+/+}$and *Vamp8*$^{-/-}$ BMDCs (left panel) and *Vamp8*$^{+/+}$ BMDCs infected with WT or *Δgp63 L. major* promastigotes (right panel) were fed with FITC-coated beads to evaluate phagosomal pH.

(C) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMDCs (left panel) and *Vamp8*$^{+/+}$ BMDCs infected with WT or *Δgp63* promastigotes (right panel) were fed with OVA-DQ-coated beads to assess phagosomal proteolysis. Data are presented as the mean ± SEM of one experiment representative of at least three independent experiments. *p ≤ 0.05; **p ≤ 0.01. See also Figure S4.

results strongly support the proposal that VAMP8 is one of the key molecules targeted by *Leishmania* to specifically alter cross-presentation.

Our data indicate that the role of VAMP8 in cross-presentation within phagosomes can be tested either in uninfected *Vamp8*$^{-/-}$ BMDCs and BMMs or/and in *Leishmania*-infected WT BMDCs and BMMs. Accordingly, we hypothesized that the phagosome functional properties observed in *Vamp8*$^{-/-}$ cells should be similar to those observed in *Vamp8*$^{+/+}$ cells infected with WT *L. major* promastigotes (a condition that degrades VAMP8). Based on this assumption, we first compared phagosomal acidification (a hallmark of phagosome maturation). As shown in Figure 4B, the phagosomal pH was significantly lower in *Vamp8*$^{-/-}$ BMDCs at all time points evaluated after phagocytosis compared to *Vamp8*$^{+/+}$ BMDCs. Similarly, the phagosomal pH was significantly lower in WT *L. major*-infected *Vamp8*$^{+/+}$ BMDCs compared to uninfected BMDCs or to BMDCs infected with the *Δgp63 L. major* mutant (Figure 4B). From these results, we concluded that the presence of VAMP8 is required to maintain a near-neutral pH in BMDC phagosomes.

The pH in the phagosome lumen influences directly the proteolytic activity and antigen processing within this organelle (Delamarre et al., 2005). We therefore determined whether the observed decrease in antigen cross-presentation in *Leishmania*-infected WT BMDCs and in *Vamp8*$^{-/-}$ BMDCs was accompanied by a defect in phagosomal proteolytic activity. Degradation of the general protease substrate DQ OVA was significantly increased in *Vamp8*$^{-/-}$ BMDCs as well as in *Vamp8*$^{+/+}$ BMDCs infected with WT *L. major*, when compared to uninfected and to *Δgp63 L. major*-infected *Vamp8*$^{+/+}$ BMDCs (Figure 4C). The processing of antigens for cross-presentation in phagosomes is also tightly controlled by the oxidation level generated through the NADPH oxidase activity. Conflicting reports either link the control of the proteolytic activity to a direct effect of the NADPH oxidase on the phagosome pH level (Savina et al., 2006) or through the redox modulation of local cysteine cathepsins (Rybicka et al., 2012). Remarkably, treatment with the NADPH oxidase complex inhibitor diphenylene iodonium (DPI) reversed the phagosomal proteolytic activity in *Vamp8*$^{+/+}$ BMDCs to the levels observed in *Vamp8*$^{-/-}$ BMDCs (Figure 4C). Treatment of *Δgp63 L. major*-infected cells with DPI also led to the levels of phagosomal proteolytic activity observed in WT *L. major*-infected cells. These observations suggest that phagosomal oxidative activity is impaired in *Vamp8*$^{-/-}$ BMDCs and in WT *L. major*-infected cells, which may be responsible for the increased antigen degradation in those cells.

VAMP8 Regulates Phagosomal Oxidative Activity

To verify the possible involvement of VAMP8 in the regulation of phagosomal oxidative activity, BMDCs that internalized dihydrorhodamine (DHR)-coated beads were analyzed by FACS for ROS production. The phagosomal oxidative activity observed in *Vamp8*$^{+/+}$ BMDCs was inhibited by DPI, indicating that it was predominantly generated by the NADPH oxidase complex. In contrast, phagosomal oxidative activity was defective in *Vamp8*$^{-/-}$ BMDCs and was not further reduced by DPI (Figure 5A). Similar to *Vamp8*$^{-/-}$ BMDCs, phagosomal oxidative activity was impaired in *Vamp8*$^{+/+}$ BMDCs infected with WT *L. major* promastigotes in a GP63-dependent manner (Figure 5A). We concluded that VAMP8 is required for phagosomal oxidative

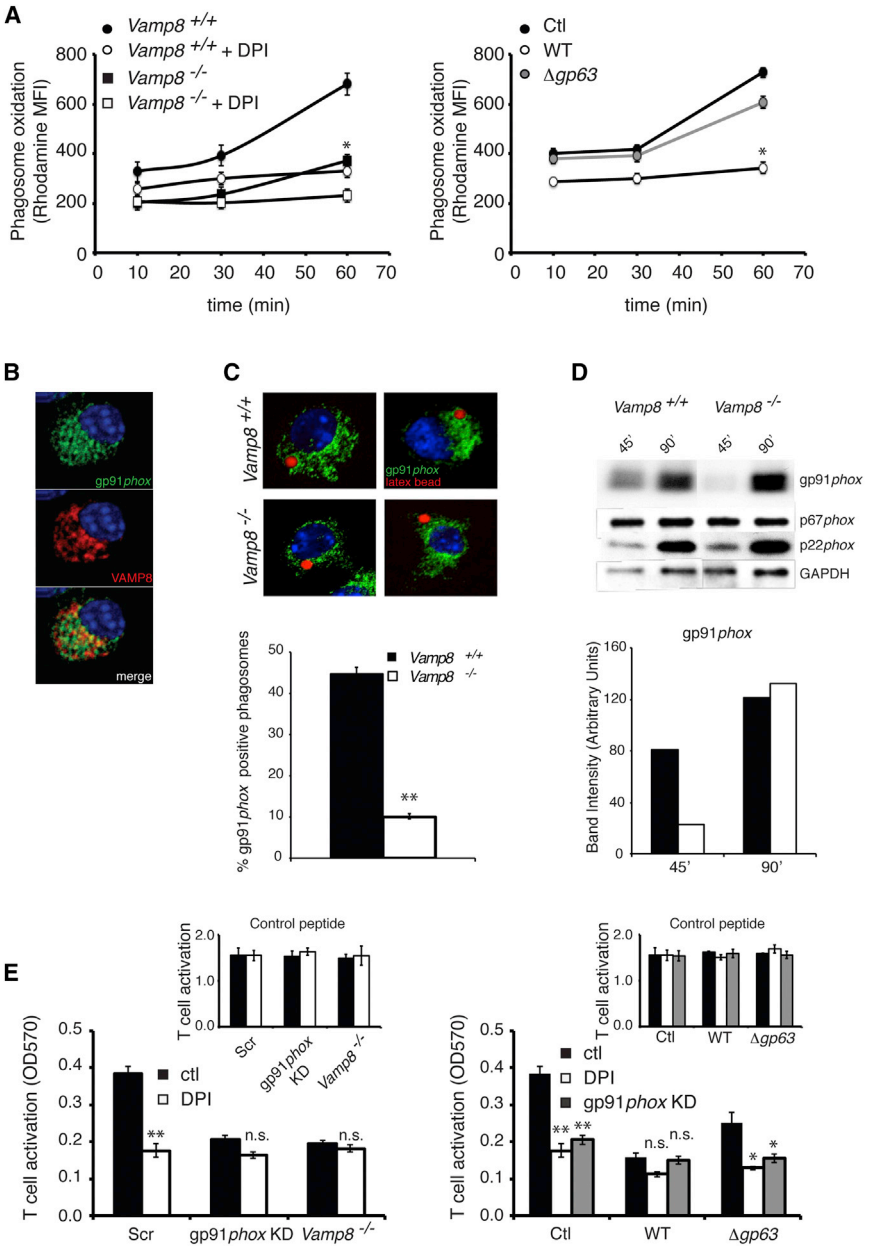

Figure 5. gp91^{phox} Is Inefficiently Recruited to the Phagosome in *Vamp8*$^{-/-}$ Cells

(A) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ cells (left panel) or *Vamp8*$^{+/+}$ cells infected with WT or *Δgp63 L. major* promastigotes (right panel) were fed with DHR-coated beads and assayed for phagosome oxidation.

(B) gp91^{phox} (green) and VAMP8 (red) partly co-localize in BMDCs.

(C) Cells were fed with fluorescent latex beads (red), and gp91^{phox} (green) recruitment to the phagosome was evaluated after 45 min. The lower histogram shows the quantification of gp91^{phox} recruitment to phagosomes.

(D) The presence of NADPH oxidase subunits in purified latex bead phagosome extracts (45 and 90 min) was assessed by immunoblots. The lower histogram shows the densitometry quantification of the gp91^{phox} immunoblots.

(E) BMDCs expressing shRNA-targeting gp91^{phox} (gp91^{phox} knockdown), nontargeting scramble (Scr), control, or *Vamp8*$^{-/-}$ BMDCs (left histogram) and gp91^{phox} knockdown or Scr BMDCs infected or not with WT or *Δgp63* promastigotes (right histogram) were fed with OVA-coated beads in the presence or not of DPI. Cross-presentation or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) was evaluated using B3Z T cell hybridoma. Data are presented as the mean ± SEM of one experiment representative of at least two independent experiments. *p ≤ 0.05; **p ≤ 0.01. See also Figure S5.

activity and that its degradation by GP63 may be responsible for the alteration of oxidative activity in WT *L. major*-infected BMDCs.

To further understand the mechanisms possibly linking VAMP8 to the phagosomal oxidative activity, we assessed whether this SNARE participates in the recruitment of the NADPH oxidase complex subunits to phagosomes. A key component of this complex, gp91^{phox}, is found in late endosomes called "inhibitory lysosome-related organelles" in reference to the ability of these organelles to regulate phagosomal proteolytic activity after NADPH complex recruitment (Savina et al., 2006). VAMP8 also localizes to late endosomes (Pryor et al., 2004). Immunofluorescence analyses indicate that VAMP8 and gp91^{phox} indeed partially colocalize in BMDCs (Figure 5B) and that in the absence of VAMP8, the recruitment of gp91^{phox} to phagosomes was strongly inhibited (Figure 5C). To analyze further the impact of VAMP8 on the recruitment of components of the NADPH oxidase to phagosomes, we purified phagosomes from BMDCs at different time points after the initiation of phagocytosis. Consistent with the results obtained by confocal microscopy (Figure 5C), we observed an inhibition of gp91^{phox} recruitment in *Vamp8*$^{-/-}$ phagosome extracts, whereas recruitment of the other subunits was not altered (Figure 5D). These results indicate that VAMP8 is required for the recruitment of gp91^{phox} to phagosomes, a process that plays a key role in the regulation of antigen processing for cross-presentation. In agreement with these observations, DPI inhibited cross-presentation in *Vamp8*$^{+/+}$ BMDCs as well as in cells infected with *Δgp63 L. major* promastigotes (Figure 5E). In contrast, DPI treatment did not further inhibit cross-presentation in either *Vamp8*$^{-/-}$ or gp91^{phox} knockdown BMDCs and WT *L. major*-infected *Vamp8*$^{+/+}$ BMDCs (Figure 5E), consistent with the fact that phagosome oxidation is already impaired in these cells (Figure 5A). To test whether our results obtained with OVA-coated beads were also relevant in *Leishmania* phagosomes, we infected BMDCs with WT or *Δgp63 L. major*-OVA promastigotes and measured OT-I T cell activation. Figures S5A and S5B show that DPI inhibited cross-presentation of *Leishmania*-derived OVA in BMDCs infected

with Δ*gp63 L. major*-OVA promastigotes, and no further inhibition was observed in WT *L. major*-OVA promastigote-infected cells. Furthermore, compared to normal BMDCs, *Vamp8*$^{-/-}$ BMDCs infected with Δ*gp63 L. major*-OVA promastigotes displayed a strong defect for the activation of OT-I T cells, and no further inhibition of cross-presentation was observed when we added DPI (Figures S5C and S5D). Collectively, our results indicate that cleavage of VAMP8 enables *Leishmania* promastigotes to inhibit cross-presentation by impairing the recruitment of gp91^{phox} to phagosomes, a key step for the control of phagosomal proteolysis. Consistently, the absence of VAMP8 resulted in an increased susceptibility to *L. major* infection in a mouse model of cutaneous leishmaniasis (Figure S5F).

DISCUSSION

In the present study, we unraveled a strategy used by *Leishmania* parasites to evade immune recognition by inhibiting cross-presentation, a process involved in the development of a protective immune response during leishmaniasis (Belkaid et al., 2002; Bertholet et al., 2006; Da-Cruz et al., 1994; Müller et al., 1991; Stäger and Rafati, 2012; Uzonna et al., 2004). We found that cross-presentation is inhibited by GP63 and is inhibited in the absence of VAMP8, supporting the proposal that cleavage of this SNARE enables *Leishmania* promastigotes to inhibit this important immune process. A recent publication showed that Sec22b, a SNARE associated with the endoplasmic reticulum and localized to the phagosome membrane, is also required for cross-presentation (Cebrian et al., 2011). Accordingly, it appears that the molecular mechanisms involved in cross-presentation are finely regulated by series of SNAREs that can be directly targeted by intracellular pathogens to evade immune recognition. The findings that bacteria such as *Chlamydia* and *Legionella* encode proteins displaying SNARE-like motifs that interfere with the function of endocytic SNAREs (Paumet et al., 2009; Wesolowski and Paumet, 2010) highlight the significance of targeting these types of molecules to manipulate the host response.

The processing of exogenous antigens for cross-presentation within phagosomes is a highly complex process that requires limited proteolytic activities regulated in part by the intraphagosomal pH and the levels of hydrolytic enzymes (Delamarre et al., 2005) (Trombetta et al., 2003). Recent reports indicated that phagosomal proteolysis, and potentially pH, is regulated by the activity of the NADPH oxidase complex (Savina et al., 2006; Rybicka et al., 2012). In the absence of VAMP8, the pH within the phagosome lumen was decreased, whereas the proteolytic activity was increased. Furthermore, we observed a severe impairment of the oxidative activity, together with an inhibition of the recruitment of gp91^{phox} to phagosomes, supporting the link between oxidation and the regulation of proteolytic activities. Interestingly, our results indicate that VAMP8 is required for the early recruitment of gp91^{phox} to phagosomes, which is crucial for the establishment of the conditions required for the processing of exogenous antigens for cross-presentation. Recent findings indicated that SNAP-23 regulates the recruitment of gp91^{phox} to phagosomes (Sakurai et al., 2012). Because VAMP8 and SNAP-23 can form SNARE complexes (Wang et al., 2004), it is possible that both act in concert to control the association of gp91^{phox} to phagosomes. Remarkably, the impairment of the phagosomal properties in cells lacking VAMP8 also occurred in a GP63-dependent way in *Vamp8*$^{+/+}$ cells infected with *Leishmania* promastigotes, supporting the concept that the cleavage of this SNARE is a key aspect of the strategy used by the parasite to subvert the immune response. In support to this proposal, we found that the absence of VAMP8 resulted in an increased susceptibility to *L. major* infection in a mouse model of cutaneous leishmaniasis (Figure S5F). Future studies will be aimed at elucidating the impact of VAMP8 on the immune response during in vivo infection. Although VAMP8 clearly plays a role in cross-presentation, other molecules targeted by *Leishmania* may also be involved in this process. Hence, we observed that cross-presentation is further decreased when *Vamp8*$^{-/-}$ BMDCs are infected with *L. major* promastigotes (Figure S5E). Whether this is related to the cleavage of SNAP-23 or syntaxin-4 (Figure S2) remains to be investigated.

We discovered that an intracellular pathogen can directly target and cleave components of the membrane fusion machinery of its host cells to inhibit antigen presentation. These findings thus significantly contribute to our understanding of the mechanisms associated with immune evasion during *Leishmania* infection. Our data clearly identified the metalloprotease GP63 as the parasite molecule responsible for the cleavage of VAMP8. Our observations that GP63-mediated cleavage of VAMP8 (and other SNAREs) and the consequential inhibition of cross-presentation are specific to the promastigote stage indicate that these events are limited to the early phase of the establishment of *Leishmania* infection. Sustained cleavage of SNAREs in parasitized cells would most likely be deleterious for host cells and for the parasites because the membrane fusion machinery must remain operational to allow for the maintenance of amastigote-harboring phagosomes and parasite replication. The mechanism by which GP63 accesses and degrades host cell substrates remains to be elucidated. The recent discovery that GP63 is a constituent of exosomes released by *Leishmania* provides a potential mechanism for the export of this molecule across the phagosome membrane (Silverman et al., 2010). Furthermore, previous studies indicated that GP63 is released from the parasite surface, exits the phagosome, and colocalizes with lipid microdomains on some of the macrophage membranes (Gomez et al., 2009). Remarkably, the finding that sets of SNAREs associate to various degrees with lipid microdomains (Gil et al., 2005) may facilitate their encounter with GP63. Further studies will address the relative contribution of other SNAREs in the fine regulation of the membrane-trafficking events regulating both the innate and adaptive immune response in antigen-presenting cells.

EXPERIMENTAL PROCEDURES

Animals and Cells

BALB/c, 129/SvJ, VAMP8 null (Wang et al., 2004), and *Rag*$^{-/-}$ OT-I T cell receptor transgenic mice (specific for the K^{b}-restricted OVA$_{257-264}$ epitope) were maintained in our animal facility. Animals were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal Care, and animal work was approved by the Comité Institutionnel de Protection des Animaux of INRS-Institut Armand-Frappier (protocol 0811-09). BMMs or BMDCs were obtained by growing bone marrow cells from female mice for 7 days in Dulbecco's modified Eagle's medium with

L-glutamine (Life Technologies) for BMMs, or RPMI-1640 for BMDCs, supplemented with 10% heat-inactivated FBS (Hyclone, Logan), 10 mM HEPES (pH 7.4), and antibiotics (complete medium) in the presence of 15% (v/v) L929 cell-conditioned medium as a source of CSF-1 for BMMs, or in the presence of 20 ng/ml GM-CSF for BMDCs. Cells were made quiescent by culturing them in the absence of CSF-1 or GM-CSF for 18 hr prior to use. RAW 264.7 mouse macrophages were cultured in complete medium in a 37°C incubator with 5% CO_2. The β-galactosidase-inducible OVA-specific $CD8^+$ T cell hybridoma B3Z (provided by W. Heath, University of Melbourne) was maintained in RPMI-1640 medium supplemented with 5% (v/v) FCS, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), G418 (0.5 mg/ml), and hygromycin B (100 mg/ml). The $OVA_{323-339}$-specific, $I\text{-}A^b$-restricted BO97.10 hybridoma (a kind gift from P. Marrack) was maintained in Kappler Marrack complete tumor medium.

Parasites

Leishmania promastigotes (WT and Δ*lpg2 L. donovani* LV9; WT, Δ*gp63*, and Δ*gp63*+*gp63 L. major* Seidman clone A2; *L. tarentolae*) were grown at 26°C in M199 medium supplemented with 20% heat-inactivated FBS, 100 μM hypoxanthine, 40 mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin, and antibiotics (*Leishmania* medium). *L. donovani* amastigotes were recovered from the spleen of hamsters infected for 6–8 weeks. *L. major* promastigotes expressing a secreted form of OVA (*L. major*-OVA) were generated by electroporating the pKS-NEO SP:OVA construct, which encodes a fusion protein containing the signal peptide of the *L. donovani* 3′ nucleotidase-nuclease fused to a portion of OVA protein (139–386) containing both MHC class I $OVA_{257-264}$- and class II $OVA_{323-339}$-restricted epitopes (Bertholet et al., 2005) (kindly provided by Alain Debrabant, FDA). Transfected parasites were grown in *Leishmania* medium supplemented with 50 μg/ml G418. For infections, promastigotes were used in the late stationary phase of growth (Vinet et al., 2009).

Reagents and Antibodies

Rabbit antibodies to VAMP8 and SNAP-23 were from Synaptic Systems. Rabbit antibodies to Syntaxin 3, VAMP3, Calnexin, and ERp57, and the mouse monoclonal to Syntaxin 4 were from Abcam. Mouse monoclonal to β-actin and rabbit anti-OVA were from Sigma-Aldrich. Mouse monoclonal antibodies to GP63 were kindly provided by W. Robert McMaster (University of British Columbia). Goat antibodies to TAP1 were from Santa Cruz Biotechnology. Rabbit antibodies to Sec61 were from Affinity BioReagents. Mouse monoclonal antibody to NSF was a kind gift from Sidney Whiteheart (University of Kentucky). Mouse monoclonal antibodies to $gp91^{phox}$, CD8α APC-H7, Vα2 PerCP, CD3 (conjugated to PE-Cy7), and CD69 (conjugated to APC) were from BD Biosciences. Purified OVA (grade VI, 99% pure), DPI, CPRG (chlorophenol red-β-D-galactopyranoside), and PMA (phorbol myristate acetate) were from Sigma-Aldrich. The SIINFEKL peptide was from AnaSpec.

Phagocytosis Assays

Complement opsonization of *Leishmania* promastigotes with BALB/c mouse serum was done as described by Vinet et al. (2009). Cells were incubated with Zym or parasites at a particle-to-cell ratio of 15:1 for different time points. For FACS experiments, cells were incubated 30 min with 1.9 μm fluorescent latex beads, previously coated with OVA. Cells were then washed in PBS, 5 mM EDTA to eliminate noninternalized beads. Cells were then cultured in complete medium for various times of chase and then labeled with an anti-OVA antibody. Phagocytosis was evaluated using FACSCalibur flow cytometer, and noninternalized beads (OVA-positive cells) were excluded.

Microscopy and Immunofluorescence

Cells were fixed and permeabilized as described by Vinet et al. (2009). Phagosomal recruitment of VAMP8, VAMP3, and $gp91^{phox}$ was quantified by immunofluorescence microscopy using an oil immersion Axio Observer Z1 microscope equipped with a Zeiss LSM780 confocal system. Results are based on at least 300 cells in each independent experiment (100 cells per each coverslip), and each experiment was repeated twice. For $gp91^{phox}$ staining, cells were visualized with a wide-field microscope (DeltaVision Elite Inverted Microscope) equipped with a CoolSNAP HQ2 camera using a 60×/1.42 NA oil objective. Images were acquired and deconvoluted using SoftWoRx Software (Applied Precision).

Phagosome Preparation

Cells were incubated for 15 min with 3.19 μm polystyrene magnetic particles, then chased for different periods of time and disrupted in homogeneization buffer. Magnetic phagosomes were removed from the postnuclear supernatant using a magnet, washed three times in cold PBS, and lysed. After 10 min at 4°C, magnetic bead phagosomes were removed by centrifugation. Phagosomes were also prepared using latex beads (Estapor) and discontinuous sucrose gradients as previously described by Desjardins et al. (1994).

Western Blot Analyses

Phagosomal and total cell lysate proteins were separated on 12% and 15% SDS-PAGE, transferred onto Hybond-ECL membranes (Amersham Biosciences), and immunodetection was achieved by chemiluminescence (Amersham Biosciences).

In Vitro Cleavage Assay

Reactions were performed in 200 μl binding buffer (PBS 1×, $ZnCl_2$ 1 mM) with 1 μg of purified protein. GST-tagged VAMP8 was incubated with 30 × 10^6 *L. major* promastigotes for various time points at room temperature (Gomez et al., 2009) and subjected to western blot analyses.

Design of shRNA and Knockdown Strategy

Specific shRNAs targeting the $gp91^{phox}$ and nontargeting scramble control were designed based on the sequences presented on the Sigma-Aldrich MISSION-RNA website. Annealed forward and reverse hairpin oligonucleotides were cloned into a modified pLKO.1-TRC1.5 vector where the puromycin-resistance gene was replaced with mAmetrine. Lentiviral particles were made by cotransfecting in HEK293T cells the shRNA-containing pLKO-mAM vector along with pMD2-VSVG, pMDLg/pREE, and pRSV-REV. Viral supernatants were used to transduce BMDCs (day 2 of differentiation), and 4 days after lentivirus transduction, cells were sorted according to mAmetrine fluorescence. Validation of the knockdown was done by real-time qPCR and western blot analyses.

Antigen Presentation Assay

BMMs or BMDCs were infected for 6 hr with WT or *Δgp63 L. major*-OVA promastigotes, or with nontransfected promastigotes. Cells were then washed and fixed for 5 min at 23°C with 1% (w/v) paraformaldehyde, followed by three washes in complete medium containing 0.1 M glycin. Purified OT-I T cells (using EasySep Mouse CD8+ T Cell Enrichment Kit; STEMCELL Technologies) were then added for 16 hr, and T cell activation was assayed by assessing CD69 expression within the $CD3^+CD8\alpha^+V\alpha2^+$ population. Activation was measured with a LSRFortessa flow cytometer.

For OVA latex bead assays, at 2 or 6 hr postinfection, 10^5 BMMs or BMDCs were incubated with uncoated or OVA-coated 0.8 μm latex beads for 1 hr of pulse, in the presence or not of DPI. Cells were incubated for a 3 hr chase, fixed with 1% (w/v) paraformaldehyde, and washed in complete medium containing 0.1 M glycin. For MHC class I and class II expression control, cells received the SIINFEKL peptide or the $OVA_{323-339}$ peptide, respectively, 30 min before the end of the chase. Cells were then cultured for 12 hr at 37°C together with 10^5 B3Z or BO97.10 cells for analysis of T cell activation. B3Z cells, which express β-galactosidase upon specific recognition of the $OVA_{257-264}$ (SIINFEKL)-H-$2K^b$ complex, were washed in PBS and lysed (0.125 M Tris base, 0.01 M cyclohexane diaminotetraacetic acid, 50% [v/v] glycerol, 0.025% [v/v] Triton X-100, and 3 mM dithiothreitol [pH 7.8]). A β-galactosidase substrate buffer (1 mM $MgSO_4$ × 7 H_2O, 10 mM KCl, 0.39 M NaH_2PO_4 xH_2O, 0.6 M Na_2HPO_4 × 7 H_2O, 100 mM 2-mercaptoethanol, and 0.15 mM CPRG [pH 7.8]) was added for 2–4 hr at 37°C. Cleavage of CPRG was quantified in a spectrophotometer as absorbance at 570 nm, reflecting T cell activation after cross-presentation. Production of IL-2 by BO97.10 was measured by ELISA (BioLegend).

Measurement of Phagosomal pH and Oxidation

Phagosome pH and oxidation were measured as described by Savina et al. (2006). Polybead amino microspheres (3 μm; Polysciences) were covalently coupled with FITC (Sigma-Aldrich) for measuring pH, or with DHR (Invitrogen) for the measuring oxidation. In both cases, beads were also coupled to FluoProbes 647 (pH insensitive and oxidation insensitive; Interchim). Cells

were pulsed with the beads for 20 min, washed, and further incubated for the indicated times before FACS analyses, using a gating FCS/SSC selective for cells that have phagocytosed one latex bead. For oxidation experiments, PMA (1 μg/ml) was added after phagocytosis. For DPI assays, cells were incubated in the presence of 10 μM DPI for 30 min before and during the 20 min pulse. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined. For pH measurement, values were compared with a standard curve obtained by resuspending the cells that had phagocytosed beads at a fixed pH (ranging from pH 4 to pH 8).

Measurement of OVA Phagosomal Degradation

Cells were incubated with 3 μm polybeads coupled to DQ OVA and FluoProbes 647 (Molecular Probes) for 20 min at 37°C and washed three times with PBS. At the indicated time points, OVA degradation was evaluated by flow cytometry with the fluorescein MFI measure normalized by the FluoProbes 647 MFI values. For DPI assay, cells were incubated in the presence of 10 μM DPI for 30 min before and during the 20 min pulse.

In Vivo *Leishmania* Infections

Mice were infected by injecting *L. major* stationary-phase parasites (5×10^6 in 50 μl) into the right hind footpad of 6- to 8-week-old animals. Footpad swelling was measured weekly for up to 10 weeks after infection using an electronic caliper as described by Gomez et al. (2009).

Statistical Analysis

An unpaired t test was performed using GraphPad software to assess whether the differences between control and infected groups were significant or not. p values <0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.06.003.

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